STIC-ILL RB125.E9

From: Canella, Karen
Sent: Tuesday, March 05, 2002 6:31 PM
To: STIC-ILL
Subject: ill order 09/819,193

Art Unit 1642 Location 8E12(mail)

Telephone Number 308-8362

Application Number 09/819,193

1. Cancer Research:
   1981 May, 41(5):1978-1983
   1988 Apr 1, 48(7):1864-1873

2. Anticancer Research:
   1987 Jul-Aug, 7(4B):781-789
   1997, Vol. 17, No. 3C, pp. 1973-1983

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Thanks!

Pathobiology 1993;61:67–76

Localization of Tenascin in Uterine Sarcomas and Partially Transformed Endometrial Stromal Cells

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Abstract

Normal mesenchymal cells within developing embryonic organs and transformed stromal cells in organs undergoing spontaneous carcinogenesis have the capacity for normal or altered expression of the extracellular matrix glycoprotein tenascin (Tn). Mesenchymal cell constituents of normal adult organs show only a very limited tendency to deposit Tn in their extracellular matrix. In the present study, we investigated whether malignant human mesenchymal cells derived from uterine sarcomas or normal human endometrial stromal cells partially transformed via transfection with selected oncogenes have the capacity to produce and deposit Tn. We reached the following conclusions: (1) compared with normal endometrial tissues, uterine sarcomas show heterogeneous, but increased, immunoreactive staining patterns exclusively within the extracellular compartment, regardless of the histologic subtype of the tumor; (2) in vitro, all normal and transfected stromal cells and cell lines examined secreted Tn into the tissue culture medium; (3) this secretory ability was not translated into morphologic uniformity, since immunoreactivity detected by confocal laser scanning microscopy was observed in only selected cell populations; (4) also, the deposition and the incorporation of Tn depended upon the density of transfected cells, and (5) double-staining experiments revealed that Tn could always be localized in close proximity to fibronectin. In summary, the production of Tn is increased in most cases of human uterine sarcoma. The capacity of stromal cells to deposit Tn in a matrix-like structure in vitro, rather than increase production of Tn, is correlated with the degree of neoplastic progression.

Key Words
Uterine stroma
Mesenchymal malignancy
Extracellular matrix
Tenascin expression
Oncogene transfection
Carcinogenesis
Confocal laser scanning microscopy

Introduction

Tenascin (Tn)/hexabrachion proteins are newly characterized glycoproteins of the extracellular matrix, which have been discovered independently in a number of laboratories [reviewed in ref. 1–4]. Recently, we have reported on the spatial and temporal constraints on the expression of Tn in the stroma of normal and progressively transformed rodent and human uterine tissue and its ubiquitous presence in the stroma of endometrial adenocarcino-

Received:
February 2, 1992
Accepted:
March 20, 1992

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1015–2008/93/0612–0067
$2.75/0

mas [5, 6]. These observations are analogous to other reported findings in mammary carcinomas of humans [7] and mice [8, 9] as well as in human gliomas [10, 11].

Two possible functions of Tn that might be related to carcinogenesis have been identified in cell culture experiments. The first is an ability to inhibit cell adhesion to fibronectin [12]. Lightner and Erickson [13] found that inhibition was effected by a steric blocking mechanism (the hexabrachion molecule covers up the cell-binding site on fibronectin), but additional mechanisms involving receptor-mediated cell signalling are also possible. Tn expression has been correlated with cell migration in embryonic development [14–17], and might be similarly involved in metastasis. The second possible function is as a growth factor, although there is still only a single report of mitogenic activity of Tn [8]. To understand the role of Tn in carcinogenesis, it appears to be necessary to recognize the factors or conditions that regulate its expression. Cell culture experiments demonstrated that Tn could be induced in chicken skin fibroblasts by culturing them in conditioned cell culture supernatants from human MCF7 breast cancer cells [18] or in the presence of TGFβ [18, 19]. Some mesenchymal tumor cells, such as the human glioma line U251-MG, secrete large amounts of Tn without stimulation.

In the present study, we use several research techniques to determine the nature and extent of Tn expression in endometrial stromal cells derived from patients, as well as human endometrial stromal cells partially transformed in vitro by the transfection of large T antigen alone [20, 21] or in combination with the c-Ha ras (EJ ras) oncogene. These findings allowed us to determine whether human endometrial stromal cells alone are sufficient for the expression and deposition of Tn, or if factors produced by epithelial cells are necessary for these processes to occur.

Material and Methods

Tissue Culture

Endometrial tissue was obtained with informed consent from hysterectomy specimens with no evidence of intrinsic endometrial disease [22]. Endometrial tissue was digested in a 0.2% collagenase solution, and the normal endometrial stromal cells (HESC) were separated from their glands by a series of centrifugations, as previously described [23]. Monolayer cultures of stromal cells were maintained in a 1:1 mixture of Opti-MEM I with 2-mercaptoethanol and RPMI 1640 (Gibco) supplemented with 1% heat-inactivated fetal bovine serum (FBS), 3% bovine calf serum (HyClone), 2 μg/ml insulin, 4 m*M* glutamine, 2× nonessential amino acids (Gibco), 10,000 U/ml penicillin, 10 mg/ml streptomycin and 2 μg/ml amphotericin B (Sigma). Experiments were performed in two different media. Cells were transferred into these media at least 1 week prior to the experiments. One of these media was based on DMEM without phenol red, and contained 5% FBS (HyClone), 2 μg/ml insulin, 4 m*M* glutamine (Gibco), 10,000 U/ml penicillin, 10 mg/ml streptomycin and 2 μg/ml amphotericin B (Sigma). The second was a serum-free medium based upon a 1:1 mixture of DMEM and F12 supplemented with 2 μg/ml insulin, 4 m*M* glutamine, 40 μg/ml transferrin, 10^{-8} *M* hydrocortisone, 2×10^{-8} *M* sodium selenite and 1 mg/ml putrescine (all Gibco).

Cell Types

Neonatal human foreskin fibroblast (HUFF) cells were from Dr. Lightner's source stock and were maintained as described above. Human sarcoma cells were obtained from clinical samples of patients undergoing surgery for uterine sarcomas at the University of North Carolina Hospitals and were maintained in long-term culture [24].

Transfection Protocol

Approximately 10^6 human endometrial stromal cells (HESC) were electrophoretically transfected with 100 μg/ml of plasmid DNA in Opti-MEM at 4.0 kV/cm² for 30 μs. Cells were transfected with a simian virus 40 (SV40) construct defective in the origin of replication, and coding for a mutant temperature-sensitive antigen (tsSV40) plasmid construct [25]. Cells were held at confluence until colonies of morphologically altered cells appeared. These were subcultured using cloning rings. After obtaining a clonal population, HESC expressing the tsSV40 large T antigen were maintained in culture at the permissive temperature for large T antigen function (33 °C), and a portion of the population transfected with an activated c-Ha ras oncogene carried by the plasmid PSV_2neo/EJ [26, 27], kindly provided by Dr. Marc Mass (US EPA, Research Triangle Park, N.C., USA). EJ ras-transfected cells were then isolated by G418 selection and maintained in culture at the permissive temperature.

Immunohistochemistry and Western Blot Analysis

Cell tpyes described above were plated onto 4-well multitest Lab-Tek plastic slides (Miles Scientific, Naperville, Ill., USA) at a density of 20,000 cells per well. HUFF, normal HESC and S7 sarcoma cells were maintained at 37 °C for 5–7 days prior to staining. Normal HESC (control) and HESC transfected with the tsSV40 construct ($HESC_{SV40}$) with or without the EJ ras oncogene ($HESC_{SV40+ras}$) were maintained at the permissive temperature (33 °C) for large T-antigen function for 3 days. One set of slides containing all three cell strains was transferred to the nonpermissive temperature (39 °C), while the other set of slides was maintained at the permissive temperature. Cells were permitted to grow at these conditions for 4 days. In some control experiments, cells were grown at the nonpermissive temperature immediately after plating.

For the immunohistochemical studies in situ, formalin-fixed, paraffin-embedded tissue from human endometrial sarcomas obtained from the University of North Carolina Hospitals was prepared and processed as described previously [5, 28].

Tn staining of the cells was performed in the following manner: cells were washed twice with PBS after removing the media, fixed in 3.7% formaldehyde in PBS for 10 min at room temperature, and subsequently rinsed in PBS. The cells were then permeabilized for 7 min with acetone at –20 °C and air dried. The cells were then incubated in 200 μl per well of polyclonal anti-human Tn, prepared as described elsewhere [29], at a 1:1,000 dilution for 1 h at room tem-

perature. Following this, cells were washed in PBS, and incubated for a further 30 min at room temperature with a 1:32 dilution of fluorescein isothiocyanate (FITC)-labelled sheep anti-rabbit IgG (Sigma). Cells were washed with PBS, mounted in media consisting of 5% n-propylgallate and 0.25% diazobicyclooctane [30] in polyvinyl alcohol [31] and observed with a Zeiss confocal laser scanning microscope (CLSM) at a magnification of × 1,260.

Double staining was performed in the following manner: the fixed and acetone-treated slides were first incubated with the polyclonal anti-human Tn antibody and secondary FITC-labelled sheep anti-rabbit IgG, as described above. After 3 washes with PBS, slides were incubated with 200 µl per well of monoclonal anti-human fibronectin (Sigma) at a 1:20 dilution for 1 h at room temperature, washed in PBS and incubated for a further 30 min with a 1:16 dilution of a tetramethylrhodamine isothiocyanate (TRITC)-labelled sheep anti-mouse IgG (Sigma). Slides were mounted as described above and examined in a Leitz or Nikon fluorescence microscope equipped with the appropriate filters.

For Western blotting, cell culture supernatants were removed from 60-cm^2 culture dishes, and 1 ml of sample buffer for SDS-electrophoresis was added. The dishes were heated in a boiling water bath and extracts applied to a 4–15% PAGE-Phastgel (Pharmacia). After electrophoresis, proteins were blotted to Nylon membranes by thermoblotting. After blocking with bovine serum albumin, membranes were incubated with the polyclonal anti-Tn antibody (diluted 1:500). The Nylon membranes were incubated with an alkaline-phosphatase-labelled sheep anti-rabbit IgG antibody or sheep anti-mouse IgG antibody, respectively (both Dianova, Hamburg, FRG), and the antigen was visualized using a picoBlue detection kit (Stratagene Heidelberg, FRG).

Tn Secretion

Secretion of Tn was measured with an ELISA as described elsewhere [29]. To prepare cell culture supernatants to be tested, approximately 40,000–50,000 cells were seeded in 60-mm tissue culture dishes (Falcon) in the presence of 4 ml of culture medium. After 4, 7, 10 and 14 days in culture, the cell culture supernatants were sampled and analyzed. In parallel, cells were trypsinized from the culture dish and counted using a Coulter counter. Amounts of secreted Tn were expressed in nanograms per milliliter of cell culture supernatant or picograms of Tn secreted per 100 cells per day.

Results

Uterine Sarcomas

One dozen cases of uterine sarcomas of differing histologic type were obtained from the Surgical Pathology files of the University of North Carolina Hospitals. They included cases who had undergone definitive surgery for malignant, mixed Müllerian tumors, stromal sarcomas and leiomyosarcomas. By immunocytochemistry, we were able to discriminate four different staining patterns in this heterogeneous group. In only 1 case (fig. 1c) were we unable to detect immunoreactivity for Tn within the tissue. In the majority of cases, we detected Tn staining either seen diffusely within the extracellular space (fig. 1d), as varying amounts of both linear and diffuse staining (fig. 1e), or as an intense flooding in the extracellular compartment, where most cells were surrounded by a band of Tn immunoreactivity (fig. 1f). In no case was staining limited to the mesenchymal tumor cells themselves. For comparison, the staining pattern in the normal endometrial stroma at late proliferative stages of the menstrual cycle (fig. 1a) was that of minimal Tn, present only around epithelial glands. It was also undetectable during the secretory phase of the menstrual cycle (fig. 1b) in 1 case of uterine sarcomas.

Transfection Studies of HESC

For the in vitro part of this study, we used normal HESC. Transfection of the tsSV40 large T antigen into normal HESC ($HESC_{SV40}$) resulted in low copies of the transfected gene being expressed, a prolonged life span and, in contrast to normal HESC, it allowed them to grow in culture medium without serum [25]. After the additional transfection of the c-Ha ras (EJ ras) oncogene ($HESC_{SV40ras}$), these cells tended to form colonies, regardless of whether they were grown at the permissive or non-permissive temperature. As shown previously, HESC transfected with the EJ ras oncogene exhibited positive-staining regions of the cytoplasm when stained with an antibody specific for the EJ ras protein product [32].

Tn Production and Secretion

The amounts of Tn secreted by individual cell types into the cell culture supernatants are shown in figure 2. This figure shows that all cells and cell lines tested were capable of producing and secreting Tn in a medium containing serum. It also demonstrates that transfected cells produce 5- to 10-fold more Tn normal HESC and neonatal fibroblasts. As can be deduced from this figure, prolongation of the life span by SV40 resulted in an increase of Tn secreted (fig. 2e), this is partially reduced after the additional introduction of the EJ ras oncogene (fig. 2f). If the amount of Tn secreted during the culture period is plotted as a function of the duration of the culture period, the amount of secreted Tn always increases with the duration of the experiment in permissive culture conditions (fig. 2a,c). However, if the amount of Tn measured in the cell culture supernatant is normalized to the number of cells per culture dish, we found that this previous observation holds for the HESC tested (fig. 2b). It does not hold for neonatal HUFF cells and transfected HESC, where the amount of Tn secreted first increases and then slightly decreases after extended culture periods (fig. 2d–f).

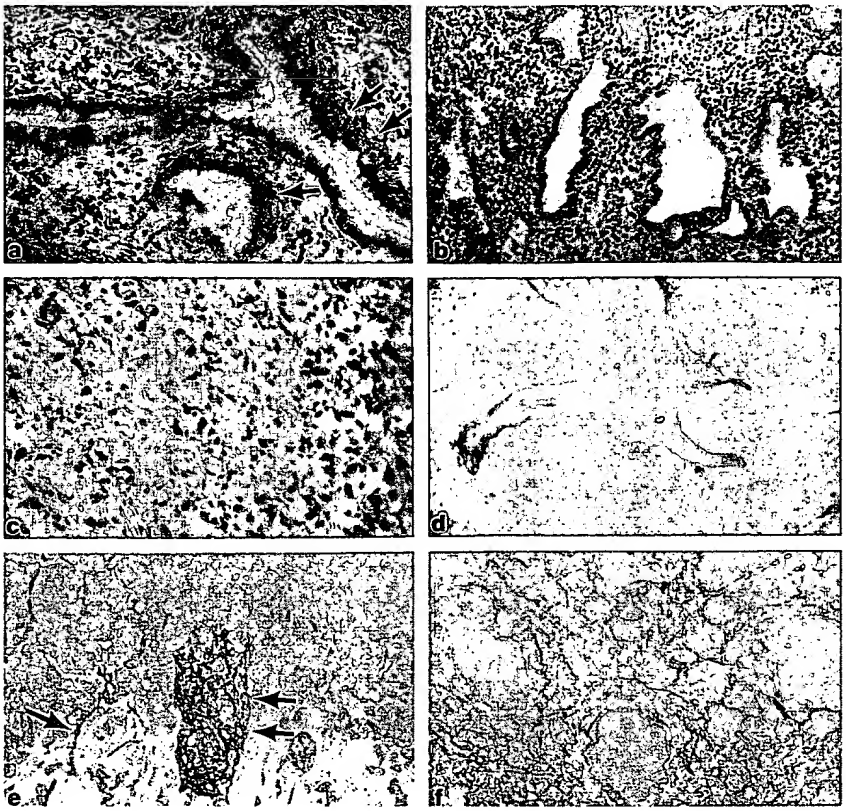


Fig. 1a–f. Tn in uterine sarcomas. In sections of normal human endometrial tissue little if any Tn immunoreactivity is detectable during the proliferative phase (**a**) but not during the secretory phase of the menstrual cycle (**b**). We were able to discriminate four different staining patterns of Tn in uterine sarcomas. In a small percentage of cases, we failed to detect Tn expression in both tumor cells and stroma (**c**). However, in another subset of cases, the extracellular space was partially filled by Tn immunoreactivity (**d**). The third group of cases could be characterized as revealing unstained neoplastic cells with varying amounts of extracellular immunoreactivity, showing both linear and diffuse staining of Tn (**e**). Clearly the largest group was characterized by an intense flooding of Tn in the extracellular compartment. Almost each cell was surrounded by a band of Tn (**f**).

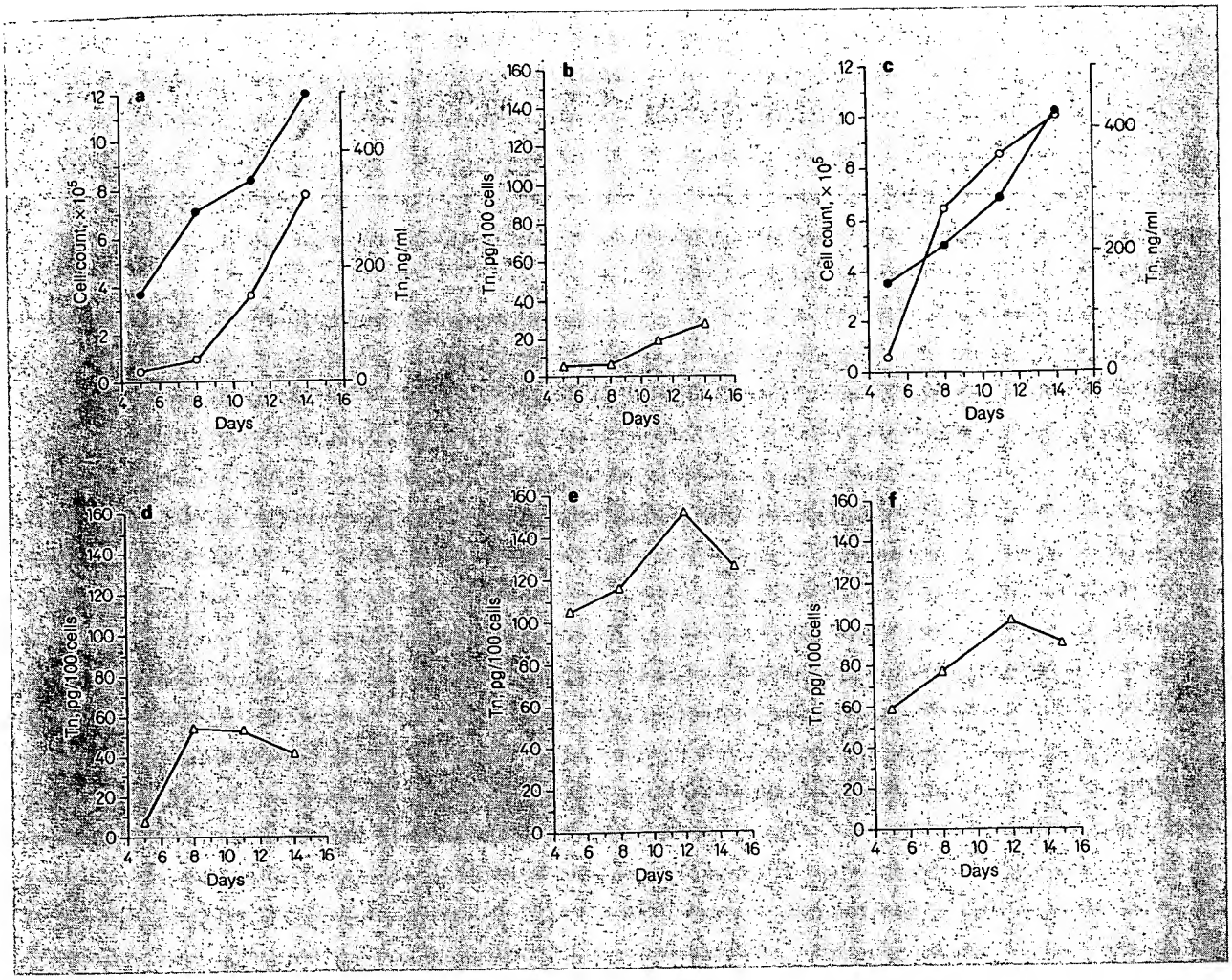
Deposition of Tn by HESC and Transfected HESC

Western blot analysis revealed that cellular Tn and Tn deposited pericellularly was present in all cell lines used in this study (fig. 3). Confocal laser scanning microscopy and immunomicroscopy revealed significant differences in Tn localization in the cell cultures examined. Normal HESC displayed granular, sparse Tn staining, predominantly in the perinuclear regions on what appeared to be rough endoplasmatic reticulum (RER) (fig. 4a). Very little deposition of Tn between cells was found. In contrast, neonatal HUFF a large amount of Tn in a pericellular localization (fig. 4b). Examination of $HESC_{SV40}$ showed that the number of cells stainable for Tn in a perinuclear localization (fig. 4c) was increased, and Tn was also found between cells. Inhibition of large T antigen function at the nonpermissive temperature (39 °C) caused cells to revert to their normal HESC-like appearance (fig. 4d). $HESC_{SV40+ras}$ at permissive (fig. 4e) and nonpermissive temperatures (fig. 4f) tended to form focal areas with extensive, fine networks of Tn between the cells. At the permissive temperature for SV40, we found that the perinuclear deposition of Tn in $HESC_{SV40+ras}$ reappeared, but, in contrast to $HESC_{SV40}$, the staining for extracellular Tn persisted (fig. 4f). S7 sarcoma cells obtained from a uterine sarcoma [24], exhibited large amounts of Tn deposition between cells, whereas the appearance of Tn in a perinuclear localization was largely absent (fig. 5, left lane).

Cell density appeared to affect Tn deposition in $HESC_{SV40}$ at 33 °C. Normal HESC, which have no capacity to grow as cell clones, usually arrest in growth at confluency without any appreciable Tn accumulation between cells. In cells expressing the tsSV40 construct the Tn staining detectable in a pericellular region increases as cells form colonies.

Spatial Distribution of Tn

If cultures of endometrial stromal cells are stained with Tn and viewed by the CLSM, the plane of localization of Tn can be analyzed. In figure 5, optical sections 1 μm apart in culture HUFF cells (right lane) and S7 sarcoma



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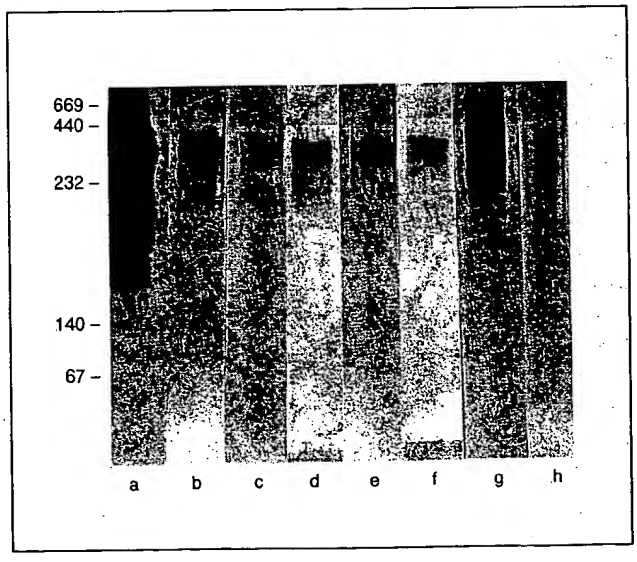

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Fig. 2a–f. Tn secretion. **a, b** Secretion by normal HESC. **a** Cell number (●) and Tn (○) accumulated in the medium at different times in culture. **b** Tn normalized against cell number. **c, d** Same analysis of HUFF cells. **e, f** Normalized Tn secretion for the transfected cells at the permissive temperature. **e** $HESC_{SV40}$, **f** $HESC_{SV+ras}$.

Fig. 3a–h. Western blot analysis of cultured cells. All cells and cell lines used in this study were capable of producing Tn. In purified forms, Tn is shown as a large band with a molecular weight of 320 kDa (**a**). In the other lanes, Tn is extracted from normal HESC (**b**), from tsSV40 transfected HESC at permissive (**c**) and nonpermissive temperatures (**d**). Tn produced by cells cotransfected with the tsSV40 large T antigen and the EJ ras oncogene are shown at permissive (**e**) and nonpermissive emperatures (**f**). Finally, Tn extracted from S7 sarcoma cells (**g**) and from neonatal HUFF is shown (**h**).

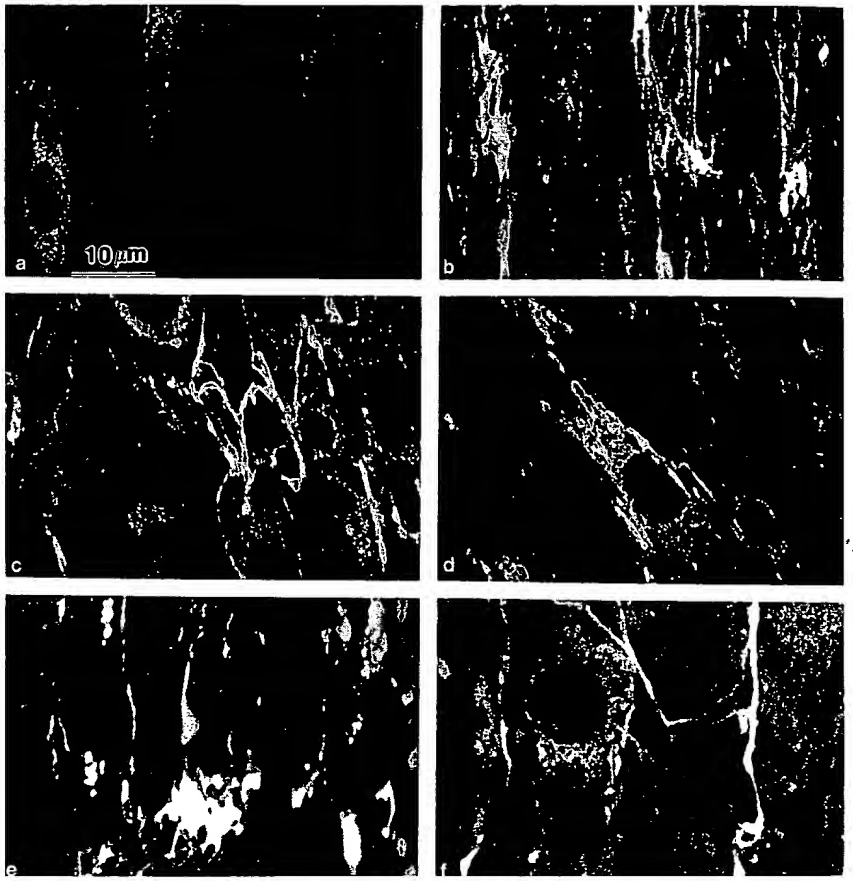

Fig. 4a–f. Deposition of TN by HESC and transfected HESC. In cultures of normal HESC, most cells did not stain, and those that did showed Tn localized in a perinuclear region (**a**). In cultures of $HESC_{SV40}$ at the permissive temperature, almost all cells showed perinuclear Tn staining, and there were small amounts of Tn between cells (**c**). At the nonpermissive temperature, these cultures were similar to HESC (**d**). In cultures of $HESC_{SV40+ras}$ at the permissive temperature, Tn is localized predominantly in fine networks between cells (**e**). At the nonpermissive temperature, these cultures still showed the matrix deposition between the cells, and also showed more prominent perinuclear staining (**f**). Neonatal HUFF cells are shown for comparison (**b**).

cells (left lane) are shown. The cells or clusters of cells are sectioned from the top of the cells towards the adhesion points of the cells on the culture dish. The amount of staining and the staining intensity appeared highest towards the midplane of the cells or cell colonies if sectioned through the whole cell layer.

Serum-Free Culture Conditions and Tn Expression

For some cell lines, serum or growth factors in the culture medium stimulate secretion of Tn in vitro in the absence of any exogenous stimulation by epithelial cells [19]. We, therefore, questioned whether the partial transformation of HESC makes them capable of expressing Tn under serum-free culture conditions by an autocrine mechanism. All cell types used grew and multiplied in the serum-free medium described above except normal HESC. All cell strains grown under serum-free conditions secreted amounts of Tn which were below the threshold of the ELISA procedure. If the serum-free cultures were analyzed immunocytochemically by confocal fluorescence microscopy, trace amounts of Tn could be visualized in cultures of neonatal HUFF cells, $HESC_{SV40+ras}$ cells, and S7 sarcoma cells. No Tn could be visualized in normal HESC and $HESC_{SV40}$ kept under serum-free culture conditions (data not shown).

Colocalization of Tn and Fibronectin

Fibronectin is a major component of the extracellular matrix of most cell cultures. We, therefore, investigated the relationship between fibronectin and Tn by colocalizing them in cultures of normal and transfected HESC using an FITC-labelled sheep anti-rabbit antibody for the demonstration of Tn, and a TRITC-labelled sheep anti-mouse secondary antibody for fibronectin visualization. In all cell lines analyzed, we did not detect the deposition of Tn without the deposition of fibronectin in close appo-

sition (fig. 6). However, the deposition of fibronectin was found to be much more ubiquitous (fig. 6c,g,i) than that of Tn (fig. 6d,h,j). Further, many fields contained fibronectin immunoreactivity alone without accompanying Tn expression.

Discussion

We have studied the occurrence, distribution and amount of Tn in mesenchymal tumors of the human uterus. We found that almost all uterine sarcomas were immunoreactive for Tn, either diffusely within the extracellular space or as a discrete band circumferentially lining every cell. In those few cases which appear to demonstrate an absence of immunoreactivity we cannot completely exclude the possibility that this is secondary to a tissue or preparative artifact. In tissue derived directly from patients, there appears to be no correlation with histologic type or 'age' of the clinical material. Admittedly, the number of cases examined is small. We have also examined Tn expression and deposition in defined cell lines and strains. We found that the capacity of stromal cells to incorporate Tn into the extracellular matrix both in vivo and in vitro, rather than their capacity to synthesize Tn, at least in vitro, is correlated with the degree of transformation of these cells. Sarcoma cells, $HESC_{SV40+ras}$ and neonatal HUFF cells deposit Tn into a matrix-like structure. $HESC_{SV40}$ exhibited this specific feature only to a lesser extent.

Normal HESC secreted about half as much Tn as HUFF cells, but failed to incorporate any Tn into a matrix-like structure. In the HESC cultures Tn was found predominantly in a perinuclear location, probably the RER and/or Golgi. A perinuclear stain, presumably of the RER, has also been described for fibronectin in mouse embryonic palatal mesenchymal cells [33] and in hepatocytes of rats with endotoxemia or sepsis [34]. In noninjured corneal endothelial cells, fibronectin was observed in RER cisternae, where it is released 48 h after injury to be deposited at the cell's Descemet's membrane interface [35]. Finally, the site of expression and deposition of the chicken integrin β_1-subunit in mouse NIH 3T3 cells appears to be predominantly on the endoplasmatic reticulum and not the plasma membrane [36].

Tn may be involved in two aspects of carcinogenesis: tumor proliferation and tumor progression. It has been shown in this manuscript and by others that Tn is closely associated with fibronectin [37]. In addition, it has been suggested that Tn possesses two contrary functions [38];

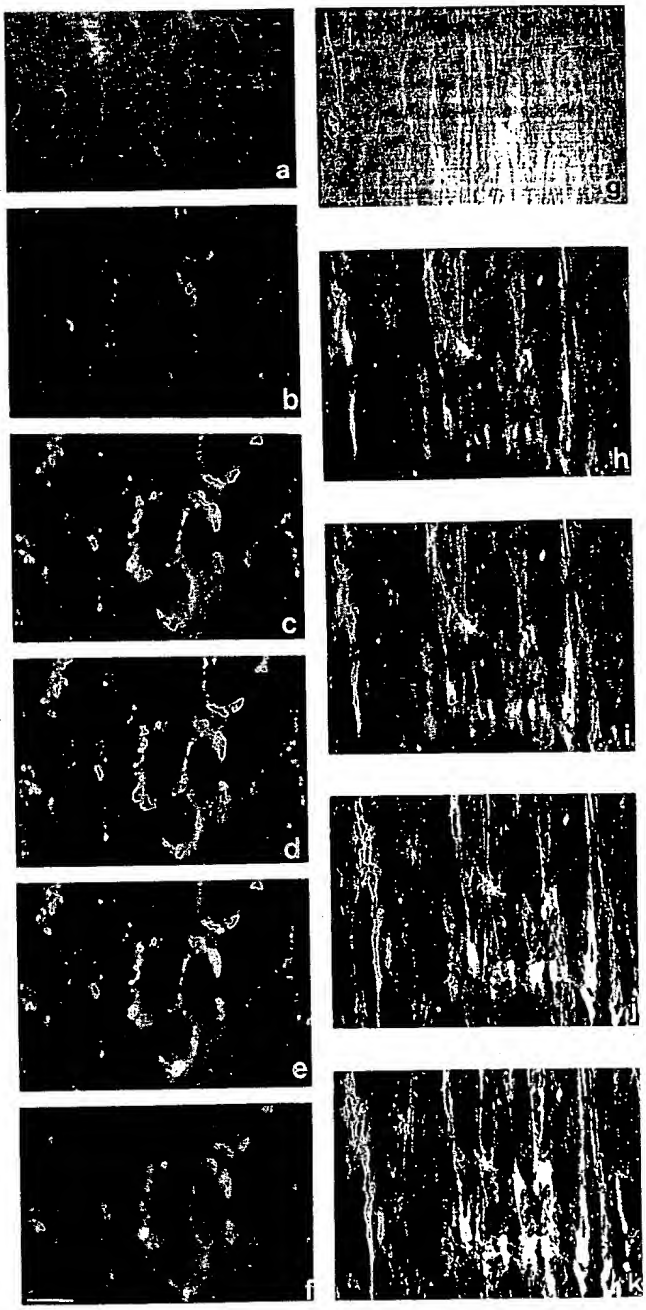

Fig. 5a–k. Optical sections of cells. Cultures of normal HUFF cells (right lane) and S7 sarcoma cells (left lane) were optically sectioned at intervals of 1 μm from the top of the cells towards the bottom of the culture dish. A differential interference contrast (DIC) image of the respective cell line is shown (**a, g**; see text for details).

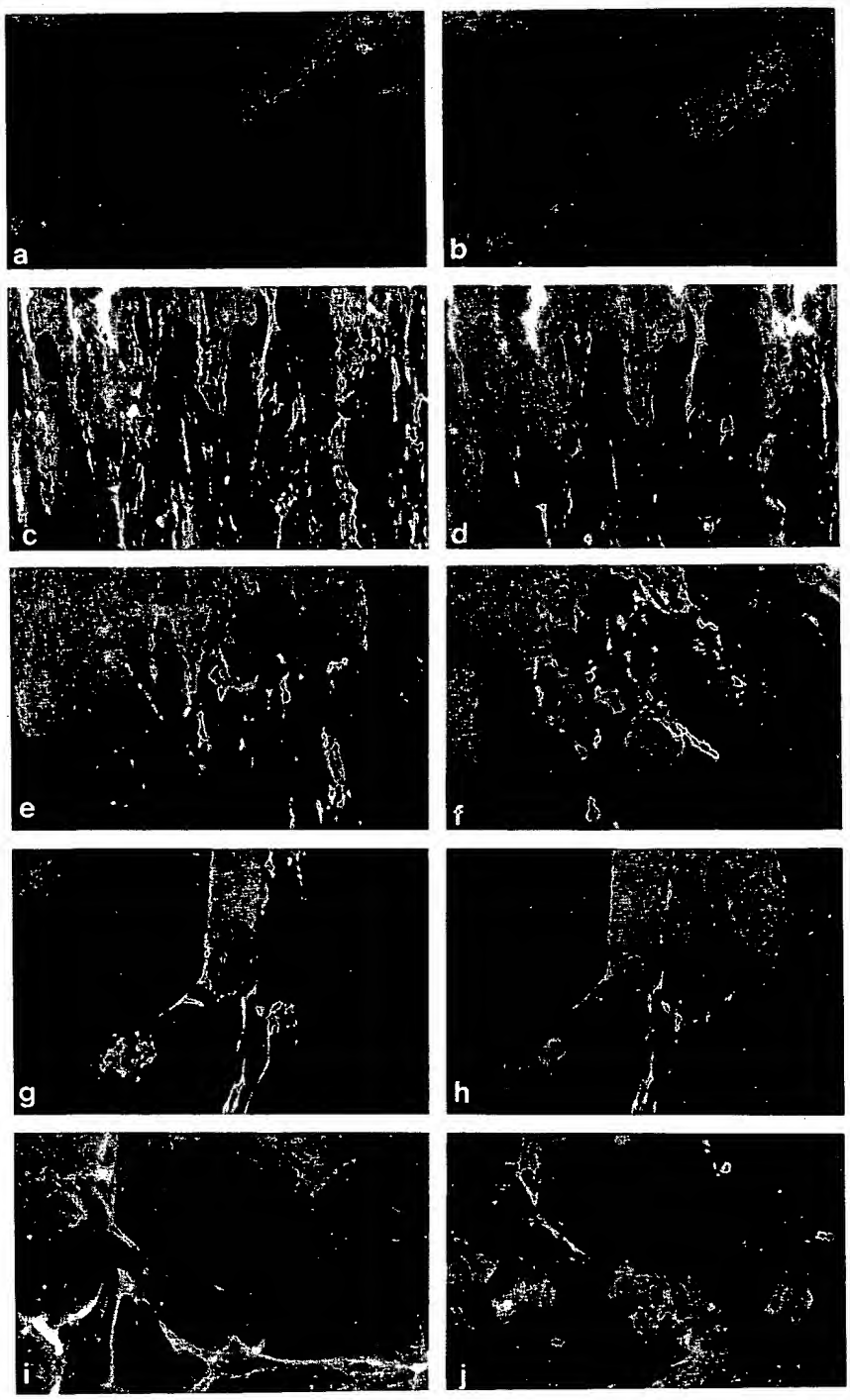

Fig. 6 a–j. Colocalization of Tn and fibronectin. Using an FITC-labelled sheep-anti-rabbit secondary antibody for detection of Tn (right column) and a TRIC-labelled sheep anti-mouse secondary antibody for detection of fibronectin (left column), we were able to colocalize both components of the extracellular matrix in all cell lines tested. Shown are normal HESC (**a, b**), tsSV40 HESC (**c, d**), tsSV40 HESC transfected with the EJ ras oncogene (**e, f**), and S7 sarcoma cells (**g, h**), HUFF cells were used as controls for comparative studies (**i, j**).

promotes attachment of cells to the Tn/hexabrachion moiety, while at the same time it facilitates the process of tumor invasion because of its antiadhesive ability to inhibit cell attachment to fibronectin. A positive correlation between cell migration and an increased appearance of Tn has already been observed during neural crest development [16]. In our studies, prolongation of the life span of cells by transfection of the SV40 large T antigen resulted in an increased production of Tn. The additional transfection of an EJ ras oncogene produced more aggressive cells that are able of invading a reconstituted basement membrane [39]. These cells produce less Tn than $HESC_{SV40}$ do, however they have a higher capacity to accumulate Tn on the cell surface.

During tumor cell proliferation, Tn may interact and participate in a feedback loop of epithelial/mesenchymal interactions. As a substrate for cell cultures, it has been shown to stimulate growth of mammary carcinoma cells in vitro [8]. Furthermore, it has been shown that production of Tn by embryonic chicken skin fibroblasts in culture could be stimulated by serum, TGFβ in serum-free medium, and serum-free medium that was conditioned by MCF7 breast carcinoma cells [18, 19]. In addition, it has been established that TGFβ in MCF7 cells can be stimulated by hormonal treatment [40]. Therefore, Tn expression might be regulated indirectly by hormones.

In conclusion, Tn appears to be a valuable marker glycoprotein for studying several aspects of endometrial carcinogenesis. Tn expression and deposition appear to coincide with the malignant transformation of stromal cells; increased amounts of Tn are found as matrix-like material between cells during neoplastic progression. Finally, the down-regulation of Tn in adult mesenchymal cells appears to reflect the stage of differentiation of the respective cells or tissues.

Acknowledgements

This paper was funded by grants of the Deutsche Forschungsgemeinschaft to G.V. (Vo 410/1-1, Vo 410/3-1) and by NIH grants CA 31733, ES 07017 (both D.G.K.), AR 38479 (V.A.L.), CA 475056 (H.P.E.) and CA 45727 (G.P.S.). We thank Dr. C. Robert Bagnell and Ms. Victoria Madden for invaluable instruction and assistance with confocal laser scanning microscopy, and Dr. Leslie Walton for his help in obtaining specimens of endometrial tissue. We are grateful to Dr. Clifford Rinehart and Ms. Tammela Butler for providing the transfected cells used in this study, and to Ms. Candice Stevens and Ms. Joanna Arcuni for their technical assistance in performing the immunohistochemical studies in the tissue. Additionally, we thank Dr. K.B. Pryzwanski for the use of her Leitz fluorescence microscope.

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